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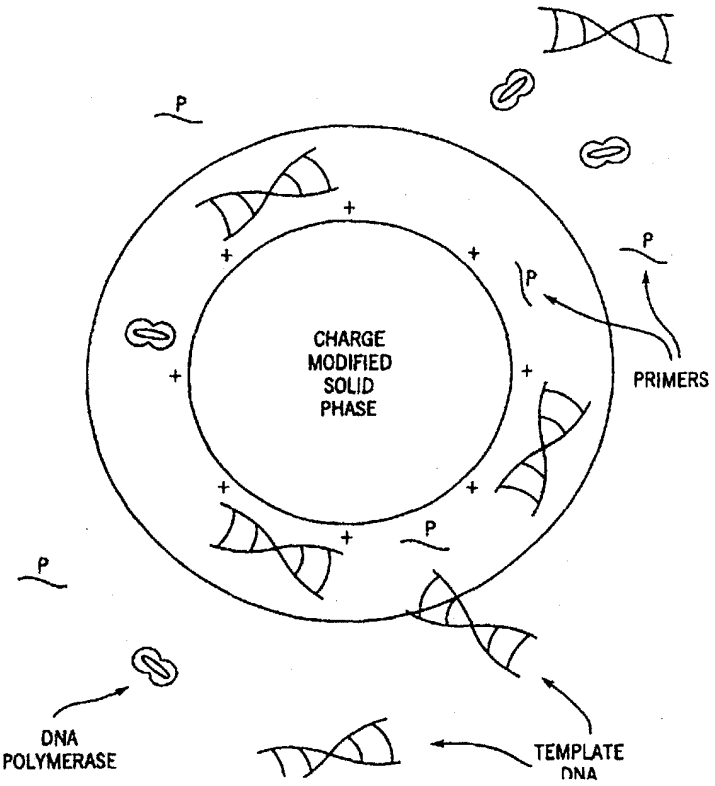
(54) Title: DEVICE AND METHODS FOR REMOTELY INDUCED THERMAL TRANSDUCTION IN CHEMICAL AND BIOCHEMICAL REACTIONS

(57) Abstract

A method and device for promoting induced thermal transduction in chemical and biochemical reactions is disclosed. In one preferred embodiment, a method of elongating a primer annealed to a DNA template is disclosed. This method comprises the first step of combining a radio frequency responsive support in an aqueous environment with a DNA template molecule, DNA primers, DNA polymerase, deoxynucleotide triphosphates and reagents necessary to amplify the DNA template. An electromagnetic field is then applied to the combination, wherein the temperature of the support will increase and the DNA template with become denatured. The electromagnetic field is removed and the temperature of the support decreases. The primer molecules anneal to the denatured DNA template and the DAN polymerase catalyzes elongation of the primer. In an especially preferred embodiment of the present invention, the support is derivatized so that the DNA template molecules are attracted to the surface of the support. The figure diagrams the relationship between PCR reagents, including the DNA template, primers and DNA polymerase, and the radio frequency responsive support.



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DEVICE AND METHODS FOR REMOTELY INDUCED THERMAL TRANSDUCTION IN CHEMICAL AND BIOCHEMICAL REACTIONS

FIELD OF THE PRESENT INVENTION

The present invention is related to devices and methods useful for reactions requiring thermal modulation. In particular, the present invention is a device useful for and methods of performing reactions that would benefit from thermal transduction, such as the polymerase chain reaction, using solid paramagnetic supports or magnetic supports to be used in combination with an energy field to generate temperature fluctuations.

BACKGROUND OF THE INVENTION

A. Chemical and Biochemical Reactions

The present invention pertains to chemical and biochemical reactions that require or are benefitted by thermal modulation or transduction. One example of such a reaction is the polymerase chain reaction (PCR). PCR has become a technological phenomenon over the last several years and is becoming a central technique employed across a wide range of disciplines and applications. The method allows for the effective analysis of trace samples, is straight-forward and robust, and is based on well-characterized and controllable chemical and biochemical reactions.

The PCR method also has several problems that have yet to be resolved. It is relatively expensive, requires a certain amount of expertise in designing optimized amplification protocols, and is dependent on inefficient instrumentation. The reliance of the method on this particular type of instrumentation necessitates the use of thermal-stable enzymes.

In essence, PCR is a set of individual reactions run in repeated sequence and facilitated by a unified buffer system that eliminates all but one type of controlled

manipulation between steps. The steps include: (1) heat denaturation of duplex DNA in the starting sample, (2) sequence-specific annealing of short oligonucleotide primers and (3) enzyme-mediated elongation of these bound primers into complementary DNA polymers. This is made possible by a buffer system that is comprised of ingredients selected for their ability to perform their primary function while not inhibiting subsequent reactions.

In PCR, applied heat is manipulated to control the onset and duration of each of these sequential steps. In a typical PCR reaction, heat is applied to the solution containing the reactants until an endpoint temperature of approximately 94°C is achieved. This reaction temperature initiates denaturation of the double-stranded DNA in the sample. The temperature is maintained for a period of time thought to facilitate efficient denaturation and then ramped down to a predetermined temperature thought to induce specific annealing of the added primer molecules to the DNA template.

Successful PCR results require significant expertise in determining the proper heat balance at the annealing step. If the operator-selected annealing temperature is too high, then the primers either do not anneal at all or anneal in too few numbers to promote good amplification. If the selected temperature is too low, then non-specific interactions are likely to occur. The determining factors include primer length, concentration and sequence. (Selected temperatures are normally between 55° and 68°C).

After the denaturation and annealing steps are completed, the temperature is then ramped up to 72°C for polymerization. This temperature is then ideal for the thermal-stable enzymes required by this approach. This is the step that is unique to the predominant PCR process. The use of a thermal-stable enzyme allows the whole process of denaturation, annealing, and

polymerization to be repeated many times over with the same enzyme. However, the same thermal-stable property that permits this enzyme to retain function after multiple exposures to the denaturation step also requires ramping up to a temperature intermediate to the denaturation and annealing steps, creating a complex and inefficient heat-chill-heat protocol.

Although many of the other DNA polymerases available have properties that might be desirable for amplification (e.g. reduced error rates, etc.) none would survive the repeated passages through the 94°C step. In fact, in the early days of this technology, investigators added fresh aliquots of enzyme prior to each polymerization step to replace enzyme destroyed during the preceding denaturation step.

B. Radio Frequency as a Temperature Modulator

Like microwaves, radio frequency waves are a form of electromagnetic energy. They also transfer energy directly into materials, primarily by the interaction of their time-varying electric fields with molecules. Radio frequency waves may be applied by connecting a radio frequency alternating current to a pair of electrodes. Between the two electrodes an alternating radio frequency electromagnetic field having a time-varying electric field component is established. When objects are placed between the electrodes in the time-varying electric field, the time-varying electric field partially or completely penetrates the object and heats it.

Heat is produced when the time-varying electric field accelerates ions and electrons which collide with molecules. Heat also is produced because the time-varying electric field causes molecules, and particularly those with a relatively high electric dipole moment, to rotate back and forth as a result of the torque placed upon them by the time-varying electric field. Most large molecules, or molecules with evenly distributed charge,

have relatively low or nonexistent dipole moments and are not very much affected by the radio frequency time-varying electric field. Small molecules, in particular with polar groups, have relatively large electric dipole moments and thus have relatively large torques exerted upon them by the time-varying electric field. In particular, highly polar molecules, like water, experience relatively large torques and as a result are rotated by the time-varying electric field, thereby transferring mechanical energy to their surroundings as internal energy or heat. Lower frequency time-varying electric fields penetrate deeply and heat objects more evenly. Relatively high frequency time-varying electric fields do not penetrate as deeply, but heat more rapidly the portions of objects they interact with.

It should be noted that a time-varying electric field is always accompanied by a time-varying magnetic field, except where destructive cancellation occurs with interference patterns. For most materials being considered here, the principal heating mechanism arises from the electric fields. These fields can cause both ohmic heating via induced ionic currents and dielectric heating via molecular stressing from the internal electric fields. For very moist materials, the presence of the accompanying time-varying magnetic field can also induce eddy-currents which can also heat the material. Also, some type of combined effect of magnetic fields and heat may occur. While the ensuing discussion is presented in context of an electric field effect, it should be understood that the effects of accompanying time-varying magnetic field are defined here for simplification as part of the electric field phenomena.

Because different materials are composed of different types of molecules with differing electric dipoles, they heat at different rates when exposed to a given time-varying electric field. For example, plastics, which are formed of very large polymer

molecules, are not heated by time-varying electric fields as rapidly as water. Metal objects may or may not be easily heated when exposed to time varying electric fields either in the radio frequency or microwave region. The high conductivity of the metal objects tends to short out the electric fields and rescatter them. As a consequence, there are many conditions where metal objects are difficult to heat, as exemplified by the metal liner in the interior of microwave ovens. On the other hand, such time-varying fields can also induce substantial currents which flow on the outside of the metal objects. Under certain circumstances heating effects will occur on the surface of the metal object which, in the case of a small needle, readily diffuses the heat into the interior. In addition, the presence of long, thin metal objects in an electric field causes enhancement of the electric field intensity near the ends of the metal objects and a diminution of shadowing of the fields near the middle. Thus, if the electric field is parallel to the axis of the metal object, strong electric fields will exist near the tips and weak electric fields will exist near the center of the rod or needle. Such field enhancements can lead to arcing and possible fires. In addition, the field suppression or shadowing of such metal objects is also an unwanted feature if the presence of a single electric field vector is relied upon in its entirety to provide the sterilization. The failure of the radio frequency electromagnetic field to penetrate the object causes surface heating only, or the opposite failure of the materials to absorb the electric field energy, causes uneven heating. In addition, similar but less pronounced absorption effects are found with water molecules. Thus, when heterogeneous mixtures have wet and dry portions, it may be seen that only the wet portions of such material would be heated.

An excellent review of some of the prior art methods for the treatment of cancer by the application of

external electromagnetic energy, capable of generating heat in intracellular particles to induce selective thermal death of cancer cells, is provided for in U.S. Pat. No. 4,106,488 to Gordon. See also U.S. Pat. Nos. 4,590,922 and 4,690,130.

Soviet Union Patent No. 1,123,705 discloses a method of sterilizing medical instruments for reuse by UHF treatment. For injection needles it discloses a final temperature of 160°C to 470°C and for acupuncture needles it discloses a final temperature of 160°C to 270°C.

Systems are also known for treatment of disposable medical waste utilizing microwaves. This system first shreds the waste, sprays the shredded waste with water, and passes the wet shredded waste through a microwave chamber designed to raise the temperature of the wet shredded waste to 205°C to sterilize it. After the sterilization step, the system compresses the sterilized shredded waste and packages it for shipment to landfills or incinerators (The Wall Street Journal, p. B33, Apr. 10, 1989).

U.S. Pat. No. 3,547,577 to Loverch discloses a machine for treating garbage by shredding, compressing the shredded garbage into briquettes, and sterilizing the briquettes with gas. After shredding the garbage is separated into magnetic and nonmagnetic portions. The sterilization step employs ethylene gas which requires temperature control. The briquettes are maintained at a temperature of about 54°C.

Attempts to kill microorganisms with radio frequency energy have generally been considered unsuccessful. In his 1980 review effects of microwave irradiation on microorganisms, Advances in Applied Microbiology 26:129-45, Chipley cites an experiment of applying radio frequency energy to bacterial and viruses which grow on tobacco. The experiment found no effect of the radio frequency energy on contaminated liquid food, there was

no showing of "selective killing effect" except when ethanol was added.

In the same review, Chipley cited numerous tests of microwaves on microorganisms and concluded that "results of tests for viability of *B. subtilis* spores also showed identical death curves compared with those obtained by conventional heat." On the other hand, however, Chipley cites several references which support the view that microwave irradiation has collateral thermal and nonthermal effects. For example, Culkin and Fung (1975) found that microbial destruction occurred at reduced temperatures and shorter time periods when the material was exposed to microwaves as compared to conventional heating methods. Wayland, et al., 1977 also demonstrated the interdependence of heat and microwave effects in the studies of spores of *B. subtilis*.

U.S. Pat. No. 2,114,345 to Hayford discloses a radio frequency applicator with electroscopic control for destroying bacteria in bottled beer and similar products. Hayford teaches an apparatus for sterilizing a series of small objects.

U.S. Pat. No. 3,948,601 to Fraser, et al. teaches the indirect use of radio frequency energy in sterilizing medical and hospital equipment as well as human waste. The reference teaches the use of radio frequency energy for heating gases, particularly argon, and exciting them so that they ionize into a plasma having a temperature of approximately 100°C to 500°C. The reference teaches that a cool plasma at a temperature of only 25°C to 50°C and very low pressure may effectively sterilize an article.

A series of investigations has been undertaken as to sterilization, especially for food. This has resulted in patents or inventions wherein the material to be treated is housed in a microwave transparent container such that the material can be heated at vapor pressures which coexist with temperatures of 120°C. These include Gray, U.S. Pat. No. 3,494,723; Nakagawa, U.S. Pat. No.

4,808,782; Stenstron, U.S. Pat. No. 4,808,783; Landy, U.S. Pat. No. 3,215,539; Utosomi, U.S. Pat. No. 3,885,915; and Fritz, U.S. Pat. No. 4,775,770.

The implication of these disclosures is that microwave heating is a valuable alternative to conventional heating means. However, the generalized heating observed would not provide differential or regionalized heating required for certain biochemical and chemical reaction.

What is needed in the art of biochemical and chemical reactions that require thermal modulation, such as polymerase chain reaction and primer extensions, is a method of performing the reaction without using conventional thermal-cycling methods and apparatus or conventional microwave heating.

BRIEF SUMMARY OF THE INVENTION

The present invention is a device and method for providing temperature modulation to chemical and biochemical reactions. In one embodiment the present invention is a method of elongating a primer molecule annealed to a DNA template. This reaction comprises the steps of combining a radio frequency responsive support, in a supportive environment, with a double-stranded DNA template molecule. DNA primers, DNA polymerase, deoxynucleotide triphosphates and other reagents necessary to amplify the DNA template would be dispersed in the aqueous phase. The support will comprise a means of attaching the template molecules. One then applies an electromagnetic field to the combination. The temperature of the support will increase and the DNA template will become denatured. One then removes the electromagnetic field, wherein the temperature of the support decreases and the primer molecules anneal to the

denatured DNA template. The DNA polymerase then catalyzes the elongation of the primer.

In a particularly advantageous form of the inventions, the support has been derivatized with moieties that attract the DNA molecule to the surface of the support.

In another particularly advantageous form of the invention, the method is repeated multiple times in order to modulate the reaction temperature multiple times and make multiple copies of the DNA template.

In another embodiment, the invention is a device to modulate chemical and biochemical reaction temperature. The device comprises a radio frequency responsive support in an aqueous environment and a means for generating an electromagnetic field.

It is an object of the present invention to provide a method and device for modulating chemical and biochemical reaction temperatures.

It is another object to the present invention to provide an improved method for performing amplification reactions, such as the polymerase chain reaction.

Other objects, advantages and features become apparent after one has studied the specification, claims and drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figs. 1A - C depict the role of the radio frequency responsive support in the present invention. Fig. 1A diagrams an electrically or radiofrequency responsive conductive support. Fig. 1B diagrams the attachment of an R group (+) capable of being utilized for reversible binding of the template. Fig. 1C diagrams the application of an electromagnetic field to the electrically conductive support.

Fig. 2 diagrams the relationship of the PCR reagents, including double-stranded DNA template, primers, and DNA polymerase, to the support.

Fig. 3 diagrams the relationship of the PCR reagents when an electromagnetic field is applied to the support.

Fig. 4 diagrams the relationship of the PCR components when the electric field is removed from the solid support and the temperature of the heat zone boundary decreases.

Fig. 5 diagrams the relationship of the PCR reagents to the solid support when the temperature decreases further and DNA polymerase begins to elongate the primer molecules.

Fig. 6A and B diagram temperature fluctuations in both standard PCR (Fig. 6A) and alternative PCR version 1 (Fig. 6B).

Fig. 7A and B describes temperature fluctuations in alternative PCR version 2 (Fig. 7A) and alternative PCR version 3 (Fig. 7B).

DETAILED DESCRIPTION OF THE INVENTION

In General

The present invention is a device useful for and methods of performing reactions that would benefit from thermal transduction and modulation, such as the polymerase chain reaction. The invention employs solid radio frequency responsive supports in combination with an energy field to generate temperature fluctuations. In one embodiment, the present invention provides a way to eliminate the requirement for the thermal-stable DNA polymerase without the necessity of replenishing the denatured polymerase.

Biochemical and Chemical Reactions

Close analysis of the PCR process reveals that the key steps in this type of amplification are most easily

controlled by applied heat. It is the manner in which heat is applied that represents the limiting factor in the current method of PCR. While it is true that temperatures in this range are needed to accomplish each of the various steps, the present invention provides an alternative way to apply heat that can be applied directly and selectively to the area where it is needed.

In the unified buffer strategy, outlined above, everything needed to support the various reactions is added in the beginning. Having all of the ingredients in the solution at the same time and using this solution to transfer heat requires that each component ingredient be stable across the whole range of temperatures.

Typically a PCR cycler uses a combination of heaters and chillers to add to or remove the thermal energy in a heat transfer liquid which is then pumped through an aluminum heat exchange block. The block features cavities for a number of specially designed thin-walled reaction tubes.

These 'tube wells' in the heat exchange block are usually filled with mineral oil by the operator to facilitate heat transfer between the block and the tubes containing the dissolved ingredients. The tubes and subsequently the buffer they contain then become the next medium for the transfer of heat to or from the vicinity of those reactants whose proximity and interaction with one another set up the necessary conditionals for the next step in the process.

For example, when denaturation is required, heat is applied indirectly by these means to two adjacent strands of duplex DNA. The thermal energy disrupts the integrity of their interaction making regions of each strand susceptible to the competitive binding potential of the short single stranded oligo primers in the mixture. However, the temperature at this point is too high to permit stable binding of the short oligonucleotides to the denatured DNA strands.

This inhibition is overcome by ramping the temperature down to a lower level. The fact that the oligonucleotides, which are themselves less stable in their interaction with the template DNA than the original DNA molecules, bind at all is due to mass action. By this I mean that the oligonucleotides are added to the mixture in higher molar proportion than the template DNA and thus eventually prevail in capturing available binding sites on the template.

The permissive temperature for annealing is much lower than the temperature required to induce efficient polymerization with thermal-stable enzymes. Therefore, the next step is to ramp the temperature back up to the higher polymerization temperature. This up-down-up, up-down-up cycling adds complexity to the cycler and its control systems. The ramping of temperatures through all the various media adds time and also requires that the entire solution be exposed to heat. These generalized temperature fluxes will unnecessarily destroy the activity of non-thermostable proteins.

The present invention employs a thermal energy flux. In the present invention, the various reaction events are still mediated by controlled heating and cooling, but there are several important differences from the traditional means. Rather than heating from the outside, the proposed system applies heat selectively at the proximity of only those ingredients that require heat. Obviously this requires regionalizing those reagents and conditions and developing a means to selectively heat in that region.

We have evaluated and characterized the interaction of solutes at the interphase that occurs between the solid and the liquid phases in various forms of chromatography. This interphase is a microenvironment with practical properties uniquely different from that of either liquids or solids. Many of these properties are exploited in commercially viable products. In nature

there are many examples of highly specific, efficient and selective actions that take place in this sort of environment.

An aqueous solution will typically still be required to contain and disperse the various soluble ingredients of the sample, but in this case it will also serve as a passive heat sink. (However other environments are equally suitable. For example, the reactants may be contained within films, waxes, glasses, organic or inorganic solvents, or superconductors or other kinds of catalysts.) A solid, radio frequency responsive component, preferably a magnetic or paramagnetic compound, would serve as a transducer to convert radiated energy into regionalized thermal energy and also as a support for a variety of available chromatography ligands. The interphase, or "heat zone boundary," would serve as the selected region where the various polymerase chain reaction reagents could interact under external control.

Hysteresis, eddy-current heating or inhibited dipole rotation might provide a means to transduce an emitted radio frequency field into localized heat. These properties can result in a so-called 'skin effect' in some materials that is localized to the surface proximity. This would satisfy the requirement for the regionalized effects that I alluded to earlier.

Often times these sources of heat are the undesirable result of 'lossy' electronic components and thus have been fairly well characterized in efforts to reduce them as much as possible. In the present invention they would be promoted for their ability to provide regionalized heat in a support which has already been demonstrated to have chromatographic utility.

Figs. 1 - 5 describe various steps of the current invention. Referring to Fig. 1A - C, the inner circles are intended to represent the cross section of a bead or strand of the radio frequency responsive material

described above. Ideally, the inner circle would be comprised of a material whose composition and dimensions are subject to our control (Fig. 1A). Preferably, attached to the surface of this material would be an R group, such as a quaternary amine, which we could then utilize for reversible ion exchange chromatography. In this specific example (e.g., Fig. 1B) the quaternary amine would charge-modify the surface so as to present a net positive charge (+). In this manner, the negatively charged double-stranded DNA template would be attracted to the surface of the support. Negatively charged primers would be inhibited from interaction with the bead through the use of a specific modulator (e.g., salt).

If the core material of the bead or strand were a tuneable transducer then irradiation with radio waves from a remote generator might be used to induce one or more of the heat generating skin effects described above.

The outer circles in Fig. 1A - C represent a heat zone boundary. This boundary will be used to describe a zone between the bead surface and the true solution phase of the aqueous system and it is within this heat zone boundary that the desired reactions will take place. In one case, this outer circle might delineate a thermocline between the heated solution near the solids' surface and the rest of the aqueous components. In another case it might be used to describe the geometric boundary of the radiant heat which is probably limited as the root function of the distance from the transducer. It might also be used to describe different convection or hydrodynamic properties between the bulk of the solution phase and the film of liquid whose proximity to the surface provides for unique properties.

In Fig. 1C, the chromatographically functionalized support (in this specific example a charge modified ion exchanging bead or strand) is being exposed to a radio frequency or other electromagnetic field which induces the solid matrix of the support to heat up at its surface

(the resulting heat flux is represented by the arrows). This is the transduction event caused by the phenomena of Eddy-Current, Hysteresis or inhibited dipole rotation discussed elsewhere.

One source of this electromagnetic field could be a device called a "Field Generator." Depending on the frequency required and the specific electromagnetic phenomenon chosen to generate the heating, this field can be in the portion of the spectrum of electromagnetic emission known as RF (radio frequencies) or might also be a low frequency alternating polarity field. The specific nature will ultimately depend on the material physics of the bead material which will have its own unique "frequency" range depending on its size and shape. Just like the size and shape of antennae change in order to best receive radio transmission of different types, e.g. short wave - microwave, etc.

Fig. 2 is provided to demonstrate various PCR reagents in context. It is intended to show that, at the beginning of the reaction, the mixture comprising the solution phase contains a relatively homogenous distribution of reagents. However, because of the composite buffer and salt configuration, in this example, duplex template DNA would tend to selectively associate and accumulate by ion exchange with the quaternary amines on the bead surface. Other attachment means are possible. (For example, attachment could be via other well known means such as interaction of biotinylated template and streptavidin-coated beads.)

This attachment provides the condition required to selectively heat the template DNA during the subsequent denaturation steps. The short primers also present would not tend to accumulate to the surface under these salt conditions. The specific salt conditions will need to be determined empirically but will fall between the minimum concentration required to inhibit binding of the oligonucleotide length primers to the charge-modified

matrix, the number of charges put on the beads (a factor in our control) and the polymerase's tolerance to salt. In other similar chromatography media this would extend from between 0 and 500 mM. The maximum salt concentration would be delineated by the salt tolerance of the polymerase used. 50-200 mM is a typical range for some polymerases. For those polymerases obtained from halophilic organisms the tolerance could be much higher.

Fig. 3 shows the effects on those molecules that happen to be in close proximity with the surface of the support when the generator is turned on and the electromagnetic field is applied to the support. The intended heat target (template DNA) is denatured by this heating. This is a necessary step to provide access for the oligonucleotide primers during the subsequent annealing step. Note that if a non-thermal-stable polymerase happens to be in the effective heating range of the bead, it could become irreversibly denatured. Also note, however, that the vast bulk of the enzyme is located outside the effective heating zone and is therefore unaffected by this heating step. The volume differential between the true solution phase and the film of liquid in the effective zone is in our control and is likely to be many orders greater for the phase outside the heating zone.

Fig. 4 depicts primer annealing. Somewhere in the temperature range, typically occurring between 55°C - 68°C, the conditions will be ideal for the sequence-specific oligonucleotide primers to find and bind to complementary stretches of the denatured template DNAs. This binding is different than that between the template and the bead and is specifically promoted by the presence of salts. Salt conditions are set so that the oligonucleotides bind only to the template. Note that under these conditions the oligonucleotides will not interact with template DNA molecules not already denatured.

Referring to Fig. 5, as the temperature reaches the ideal range for a particular enzyme being used, the conditions will be sufficient for DNA polymerization. Because two different primers are typically used, both strands of the DNA template will be copied. This means that for every DNA molecule affected two new duplexes will be formed. These steps are initiated by the interaction of bound polymerase which will have migrated in from the surrounding solution. This association is limited by diffusion rates, concentration and the volume differentials involved.

Figs. 6 and 7 show the necessary heating and cooling profiles for the various amplification procedures discussed above. These are the control steps and therefore represent instrument programming considerations. Figs. 6 and 7 show both the standard method currently employed for PCR thermocyclers (Fig. 6A) and a selection of possible versions for the system of the present invention (Fig. 6B, Fig. 7A and B).

The standard PCR protocol involves a step-up, step-down, step-up thermal sequence. Because of the thermal masses involved and because of the indirect nature of heating this requires measuring these steps in minutes. The programming controls for this system are complex and require significant knowledgeable input on the part of the user. Since existing programs call for specific endpoint temperatures it is up to the operator to calculate or guess at the ideal temperatures for annealing.

This has led to problems with the quality of results and the number of trials necessary to obtain a successful result. The present invention only requires pressing a 'run' button. The proposed heat profile is consistent with each of the steps described above, and especially for the annealing step which benefits when approaching the ideal temperature for primer binding from the high end. As a result, the protocols described here would

probably be measured in seconds instead of minutes. Thus, many more cycles may be possible in less total time.

In its simplest form, the method of the present invention might have only one heating step per cycle as opposed to two for the standard method. Heating could potentially occur in a fraction of a second and heat loss might be accomplished through natural heat decay to the environment. Passive control might be improved by using specialized coatings on the beads to affect the rate of heat loss and, thus, to provide even more effective processing. The bulk solution itself might be used as a heat sink without the accumulation of too much heat. If this is possible it might eliminate the need for coolers altogether.

Fig. 6B diagrams this heating protocol. Because the three steps (denaturation, annealing and polymerization) are done in a sequence with each requiring a lower temperature than the last, far less control may be required. Because different materials have different heat decay properties, heat may be added to the system at more than 1 point during each cycle. For example, see Fig. 7A.

In Fig. 7A, the length of time that the sample ingredients experience a particular temperature range is extended by applying some heat throughout the entire process. Since the sequence of events calls for diminishing amounts of heat all we need to do is to delay the decline. This might be done by reducing the power output from the generator or by intermittent full power pulses occurring with ever-diminishing frequency.

Fig. 7B shows a much more sophisticated profile in which the various physical and biochemical demands of this multistep process are taken into account. It may be determined experimentally that a particular step (eg. denaturation) can only be performed efficiently if the beads are heated for several seconds. Additionally,

thermodynamics and diffusion rates might require that annealing take place over a longer period than that allowed for by natural heat loss. And, it is entirely possible that a particular enzyme will require certain minimum times in order to maximize amplification of the template. This accounts for the shallowed slopes depicting the time a sample will be exposed to the temperatures that promote annealing and polymerization.

Typically, one would perform a PCR reaction with 25 - 35 cycles, although the optimum number of cycles in a PCR application depends on the amount of starting material, the sensitivity desired and the length of time one is willing to wait. 20 - 30 cycles are probably a minimum number.

EXAMPLES

1. Combining Reactants Process Summary - Prophetic Example

Template DNA and a set of oligo nucleotide-length single-stranded primers are added to a "Master Mix" containing the necessary support reagents for DNA polymerization.

Suggested Master Mix - Table 1	
A core master mix containing the normal ingredients necessary for polymerization may be supplemented or otherwise optimized for transduction heating. It will minimally comprise:	
dNTPs	Building blocks for newly synthesized DNA
DNA polymerase	Either non-thermostable or thermostable
Buffer(s)	Potential modulator for the process
Detergent(s)	Potential modulator for the process
Salt(s)	Potential modulator for the process

The samples consisting of primers, template and master mix are then added to tubes, or 96 well trays,

containing a chromatographically functionalized, radio frequency responsive, transducible support (e.g. ion-exchanging paramagnetic beads or strands).

The tubes or trays are then placed in a radio-frequency field generator programmed to perform a controlled series of thermal induction steps that selectively generate heat at the functionalized surface of this support. In one embodiment, this device will also control the duration of heating and rate of heat loss from the support.

2. Transducible Chromatographic Support

A spherical, or preferably stranded, radio frequency responsive, metallic support is proposed with a set of particular paramagnetic or electronic properties which can be remotely manipulated to generate localized heating on the surface of the support. Thermal energy would be generated by a process of remotely controlled "Skin-Effect" or "Hysteresis Heating." These phenomena are known to be modulated by radio frequency or other electromagnetic field emissions.

Examples and properties of typically suitable supports are described below in Table 2. Two metallic supports, magnetite and chromium dioxide, are compared to PBS and H_2O.

The data described in Table 2 were obtained in experiments designed to characterize various materials for radiofrequency response. Magnetite (Fe_3O_4) and chromium dioxide (CrO_2) were selected to represent magnetically responsive materials that might normally be used as the magnetic cores of supports employed in separation applications. Water, PBS and TE were selected to represent some of the most commonly encountered liquid solvents for chemical and biochemical reactions. (The data for TE is not represented in Table 2.) The range of frequencies shown are for those cases where one or more of the materials tested showed evidence of radio

frequency response. Lower frequencies (in the KHz range) resulted in little or no RF response for the materials tested in the form of transduced heat even 5W of power was applied for 2 to 6 minutes. Conversely, at much higher frequencies (greater than 2.0 GHz) one can expect to see the generalized heating representative of traditional microwave applications. The output power and duration were empirically determined.

Under these conditions both magnetite and chromium dioxide powders showed significant heating. Several peaks were observed over these frequencies which differed somewhat in the intensity of heating between the samples tested. Surprisingly, the PBS and water samples showed much less heating. This is surprising because at higher frequencies these materials would heat significantly as well.

It should be noted that the mean values obtained in Table 2 are for the full range of wavelengths tested including numerous peaks and valleys. Therefore, the "Rise over H_2O" values cited in the table are significantly lower than for the peak values.

The peaks are given for each of the materials tested in the middle portion of the table. From these data it is apparent that both magnetite and chromium dioxide heat much more readily in response to these RF wavelengths than does water or PBS. This is evidence in support of selective heating.

These differences in RF response were further validated by subsequent statistical analysis. Since the data cited are for the observed range of RF response they do not follow "normal" distribution and, because we wanted to compare more than 2 sets of data, Sigma Stat (Jandel Scientific Software) determined the best method of comparison to be One Way Anova on Ranks (Kruskal-Wallis). This test indicated that the different sets are with a high degree of confidence representative of at least two different sample populations. The calculated

probability that we have incorrectly concluded that there is a true difference in the median values of the data sets is $P=1.10x10^{-29}$.

Running a multiple, pairwise comparison test (Dunn's test) indicated that the values obtained for water, PBS and TE are not significantly different from one another. Additionally, the values obtained for magnetite and chromium dioxide are also not significantly different from one another. However, the temperature data obtained from the set of substances including water, PBS, and TE are significantly different from those obtained from the set of substances comprising magnetite and chromium dioxide. The probability that we erroneously concluded that differences truly exist between these 2 sets of substances is $P<0.05$ for the set of 10 pairwise comparisons run.

These conclusions were further verified by comparing each of the possible 10 pairwise sets using a Mann-Whitney rank sum test (a type of t-test). This resulted in $P<0.000001$ as the resulting probability for falsely concluding that differences exist in the thermal transduction between the (CrO_2, Fe_3O_4) set and the (water, PBS and TE) set.

Although other wavelengths may also result in differential transduction in other circumstances, the data for the reported wavelengths have revealed selective transduction.

Selective Transduction in Various Substances - Table 2

(3 mW at 800 to 1100 MHz for 15 Seconds)

Magnetite		CrO_2		PBS		H_2O	
Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
46.3°C	13.1°C	44.1°C	13.0°C	21.5°C	7.82°C	18.6°C	3.5°C
Rise Over H_2O+27.7°C		Rise Over H_2O+25.5°C		Rise Over H_2O+2.9°C		Rise Over H_2O+0°C	
Peaks:		Peaks:		Peaks:		Peaks:	
Frequency	Temp.	Frequency	Temp.	Frequency	Temp.	Frequency	Temp.
840 MHz	68.8°C	840 MHz	68.1°C	840 MHz	34.3°C	840 MHz	22.3°C
870 MHz	53.9°C	910 MHz	49.0°C	930 MHz	38.5°C	900 MHz	23.4°C
960 MHz	60.1°C	960 MHz	65.7°C	950 MHz	36.6°C	950 MHz	26.3°C
1020 MHz	85.2°C	1010 MHz	62.5°C	nd	nd	nd	nd
1070 MHz	55.7°C	nd	nd	nd	nd	nd	nd

Note: mW=milliwatts, MHz=megahertz, CrO_2=chromium dioxide, PBS=phosphate buffered saline, nd=not detected (not determined).

A suitable material would typically be functionalized at its surface with one of a variety of specialized ligands that promote reversible binding of DNA to the surface of the support. The efficiency and specificity of the binding interaction could then be controlled by the potential modulators described above and by the heating process itself.

3. Instrument

A preferable device useful in the method of the present invention is a programmable RF field generator capable of time controlled heating of the functionalized surface of the support and, thus, those components of the reaction that specifically interact with the support.

In one such embodiment, power converted by an AC/DC transformer is supplied to a frequency generator which is used to tune specific wavelengths through specially wound field generator coils. The fields from these coils are adjacent to and impinge on samples placed in their proximity. The specific field strengths and frequencies

can then be manipulated to remotely induce thermal transduction.

Heat induction occurs only so long as the field is activated and cooling occurs through natural heat decay from the coated or uncoated surface once the emissions cease or by controlled heat loss by reduction in the power, duration or frequency of the emitted field.

Repetitive heating and cooling steps will be controlled for total time and number of cycles.

4. Principles of Operation

The following principles of operation describe parameters and reaction choices that can be utilized in the creation of an optimized version of the method employing ion-exchanging paramagnetic beads or strands as described above.

Table 3 - Principles of Operation
Efficiency of binding is inversely related to specificity of binding.
Ion Exchange is inhibited by increasing salt concentration whereas hydrogen bonding is enhanced.
Some salt is necessary for DNA polymerization.

Because of these relationships it is possible to describe a salt concentration range that can:

1. Support effective DNA polymerization and amplification.
2. Promote direct ion exchange interactions between large duplex DNAs (template) and the surface of the support.
3. Inhibit small single-stranded DNAs (Primers) and the other temperature-sensitive components of the Master Mix from direct interaction with the functionalized support.

4. Allow indirect interaction between primers and the support by specific hydrogen bonding through the ionically bound template.

5. Prophetic Embodiment

In one prophetic embodiment, magnetite beads will be coated with streptavidin and combined with biotinylated duplex DNA. Because this template DNA has only one biotin group attached to the 5' end of one strand, the interaction with the streptavidin coated beads occurs through one strand only. The complementary strand is thus free to dissociate from the bound strand through thermal denaturation.

Once washed, only specifically bound duplex DNA will remain associated with the magnetic beads. These beads will then be combined with dNTPs (including fluorescent dCTP) primers, buffers and a primer-dependent DNA polymerase and heated. After heating, the sample will be cooled to allow annealing of the primers to the bound strand. At this point the sample is heated or allowed to cool to an intermediate temperature consistent with the requirements for the selected enzyme to allow polymerization (if any) to occur. Because the polymerase is primer-dependent, any ensuing evidence of fluorescence associated with the beads over and above that seen in template-minus control reactions will indicate that annealing of the primers to the template (a necessary prerequisite for polymerization) had been accomplished, and in turn that denaturation (a necessary prerequisite of annealing) of the otherwise stable duplex molecule had in fact occurred. This will demonstrate that the reaction is not blocked by any component when used under external heating conditions and is promoted under internal heating conditions.

CLAIMS

We claim:

1. A method of elongating a primer annealed to a nucleic acid template, comprising the steps of

(a) combining a radio frequency responsive support in an aqueous environment with a nucleic acid template molecule, primers, nucleic acid polymerase, nucleotide triphosphates and reagents necessary to replicate the template,

(b) applying an electromagnetic field to the combination,

wherein the temperature of the support will increase and wherein the template will become denatured, and

(c) removing the electromagnetic field, wherein the temperature of the support decreases and the primer molecules anneal to the denatured template, and wherein the polymerase catalyzes elongation of the primer.

2. The method of claim 1 wherein the support has been derivatized with a moiety capable of attracting a nucleic acid template molecule to the surface of the support.

3. The method of claim 1 wherein the nucleic acid is DNA.

4. The method of claim 1 wherein the method is repeated at least 2 times.

5. The method of claim 4 wherein the method is repeated at least 40 times.

6. A device capable of providing temperature cycling conditions comprising a radio frequency responsive support in a liquid environment and a means for producing an electromagnetic field.

7. A method for manipulating the temperature of a biochemical or chemical reaction comprising the steps of

(a) combining a radio frequency responsive support with reactants,

(b) applying an electromagnetic field to the combination,

wherein the temperature of the support will be modulated, and

(c) removing the electromagnetic field,

wherein the temperature of the support will be modulated.

8. The method of claim 7 wherein the radio frequency responsive support comprises a moiety capable of attracting at least one of the reactants.

9. The method of claim 7 wherein the temperature of the support in step (b) increases.

10. The method of claim 7 wherein the reactants in step (a) are combined in a liquid environment.

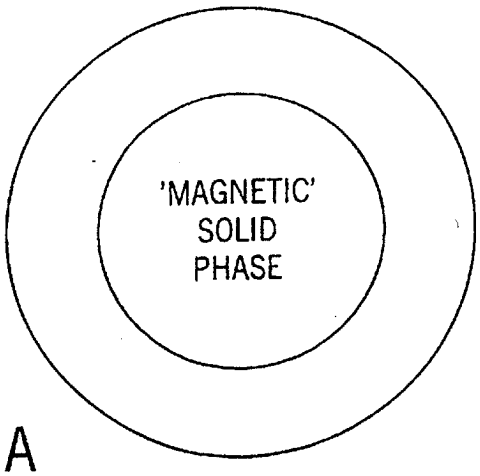


FIG. 1A

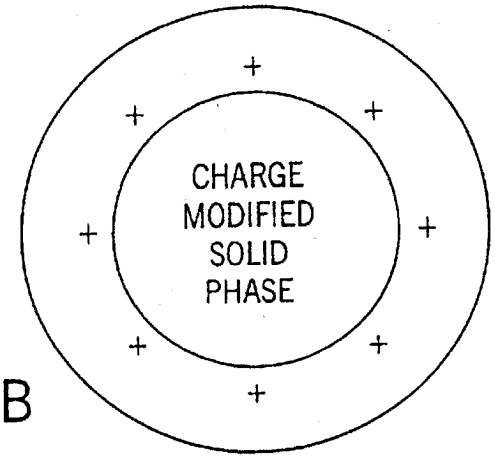


FIG. 1B

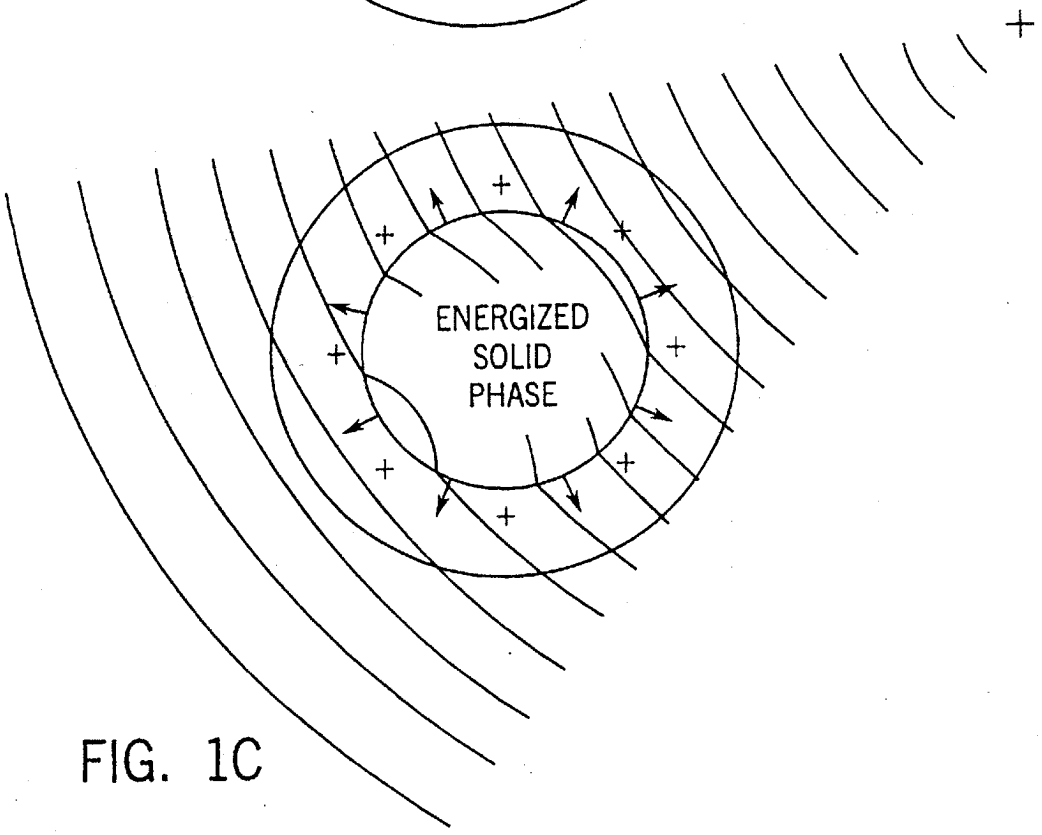
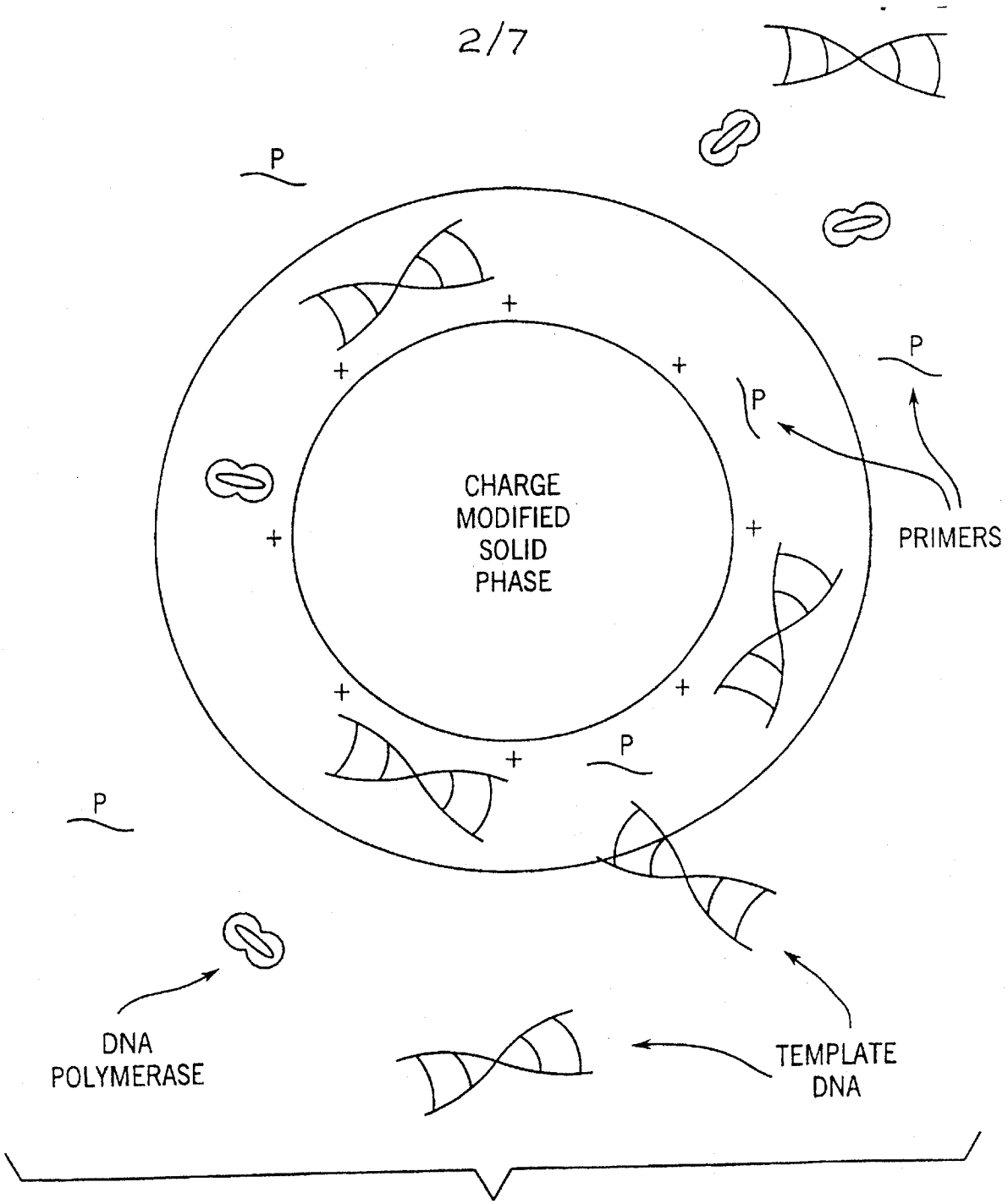


FIG. 1C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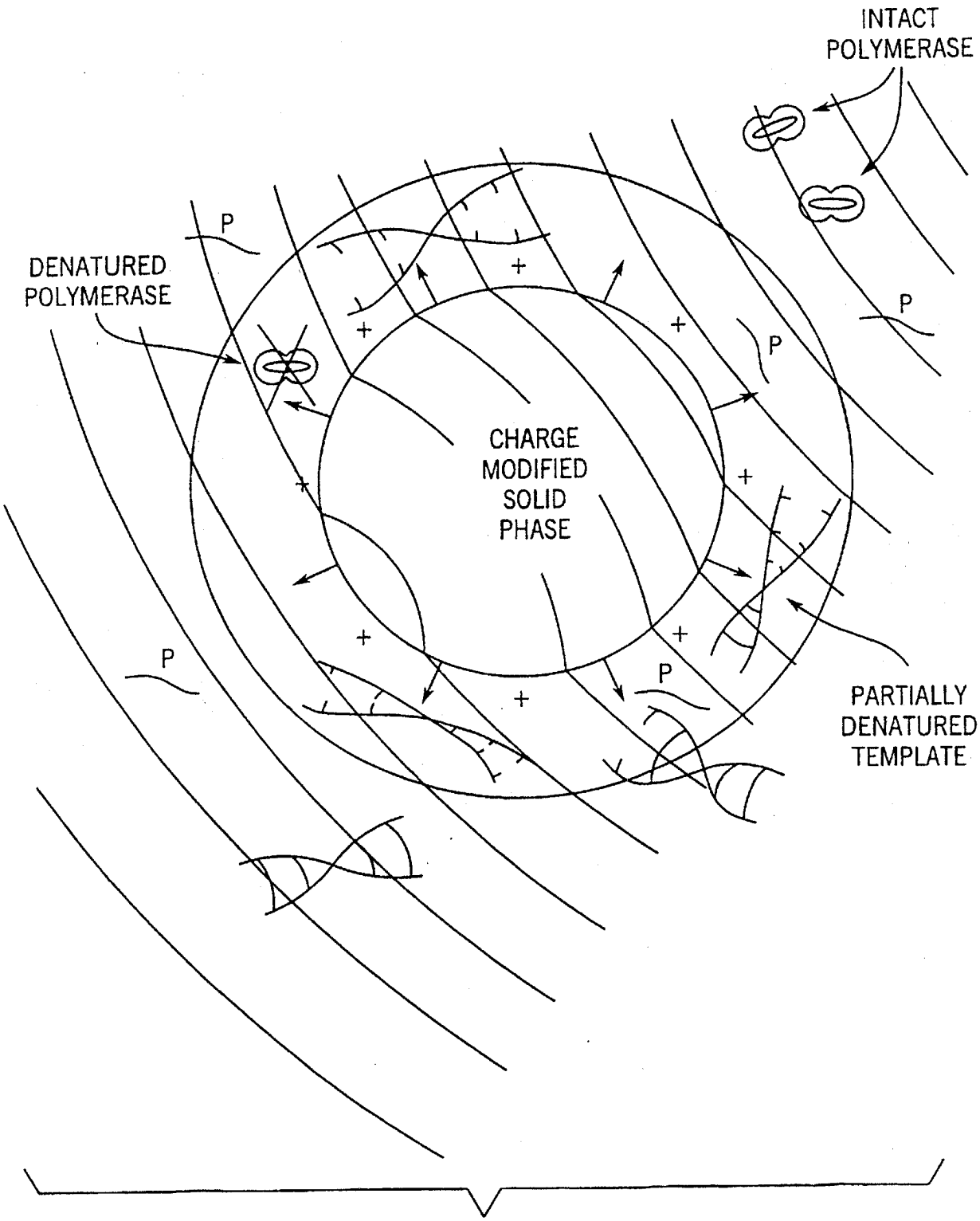
DNA
POLYMERASE
TEMPLATE
DNA

FIG. 2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DENATURED
TEMPLATE

FIG. 3

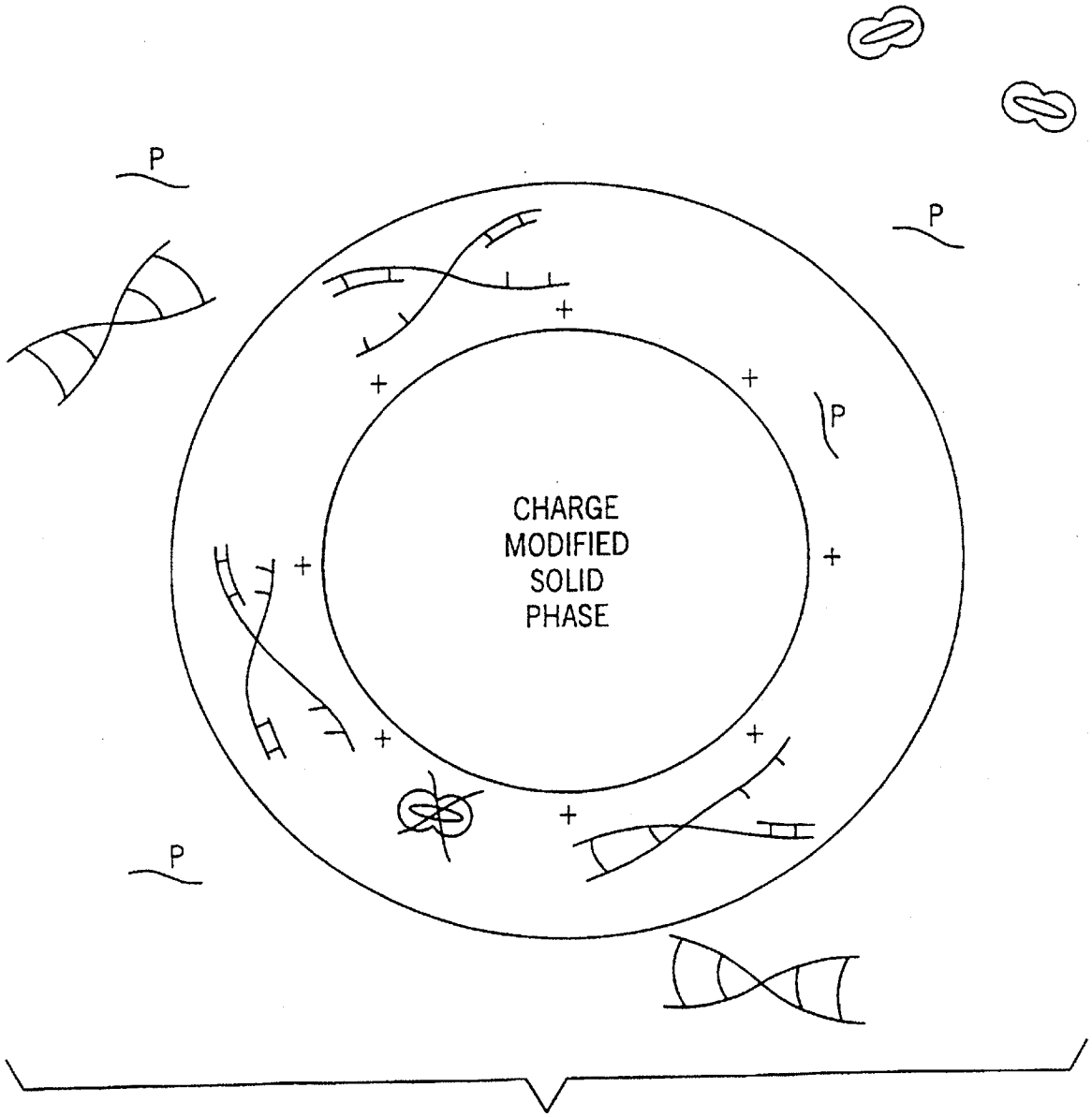


FIG. 4

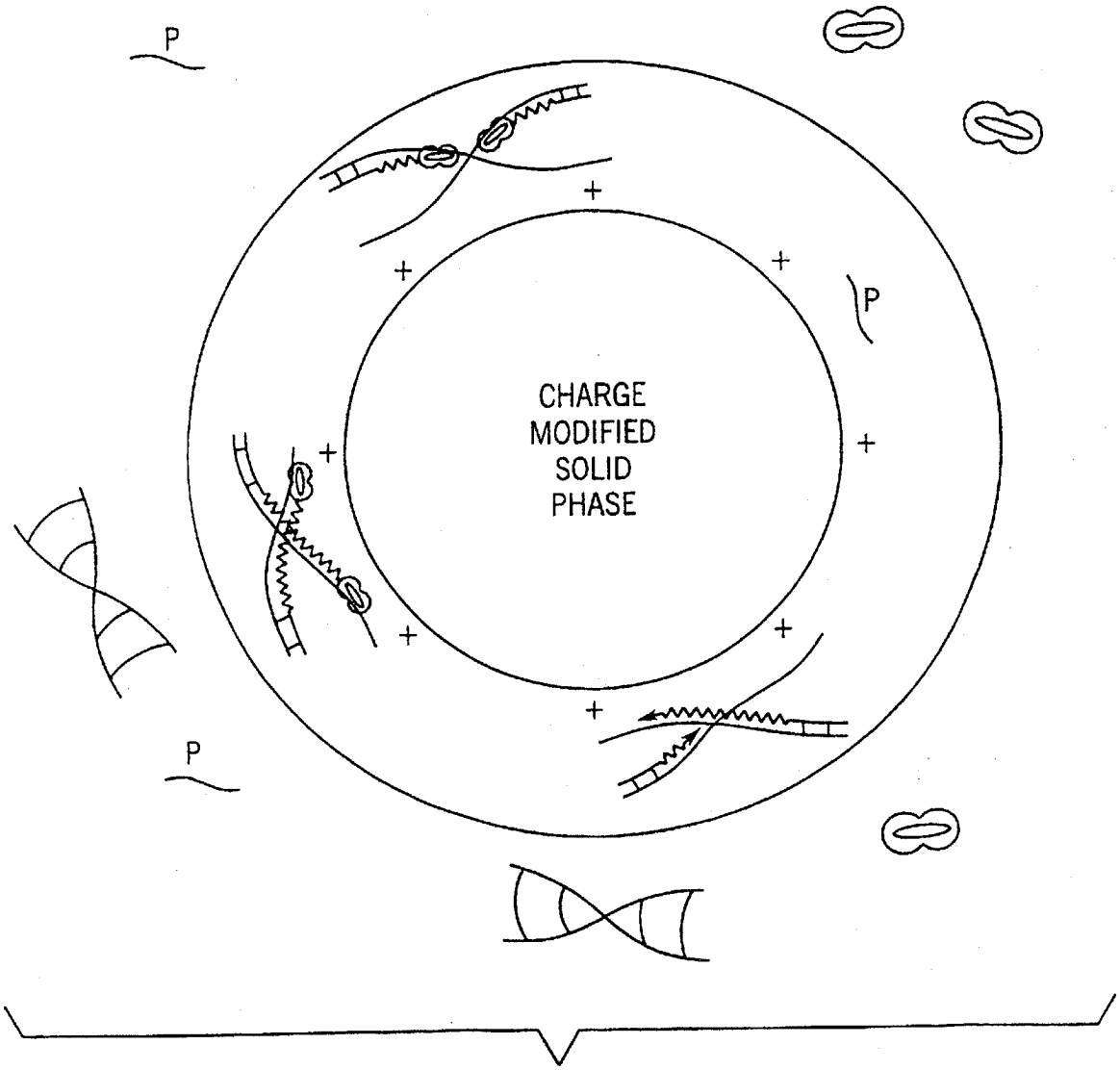
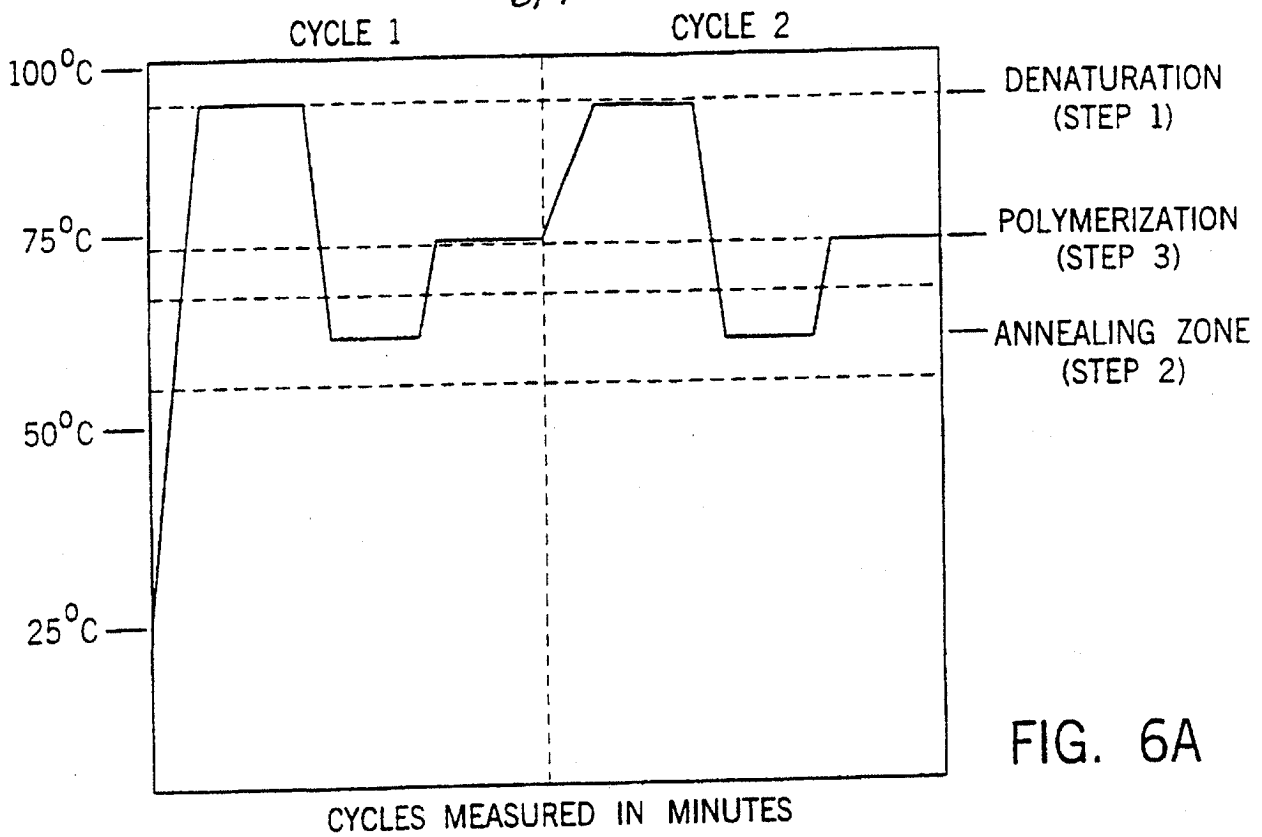


FIG. 5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CYCLE 2
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50°C
25°C
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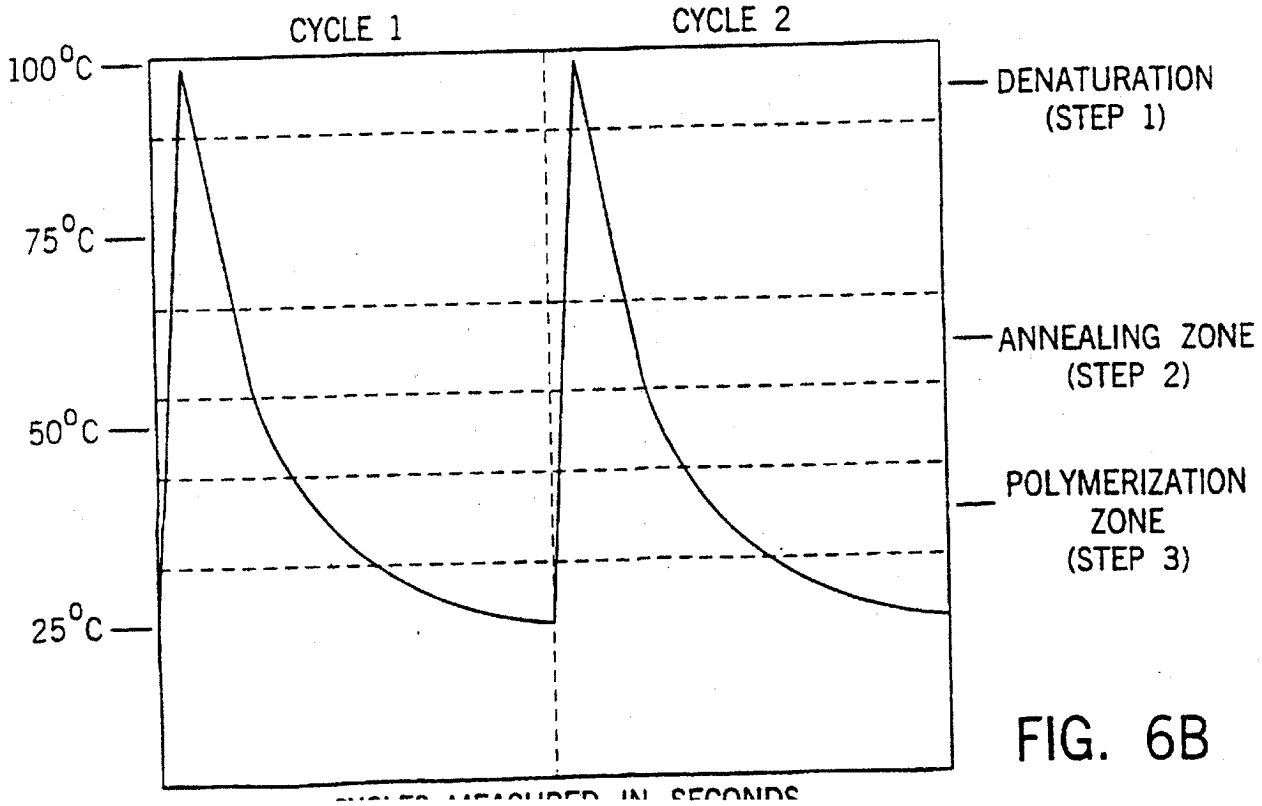
POLYMERIZATION (STEP 3)
ANNEALING ZONE (STEP 2)
CYCLES MEASURED IN MINUTES

FIG. 6A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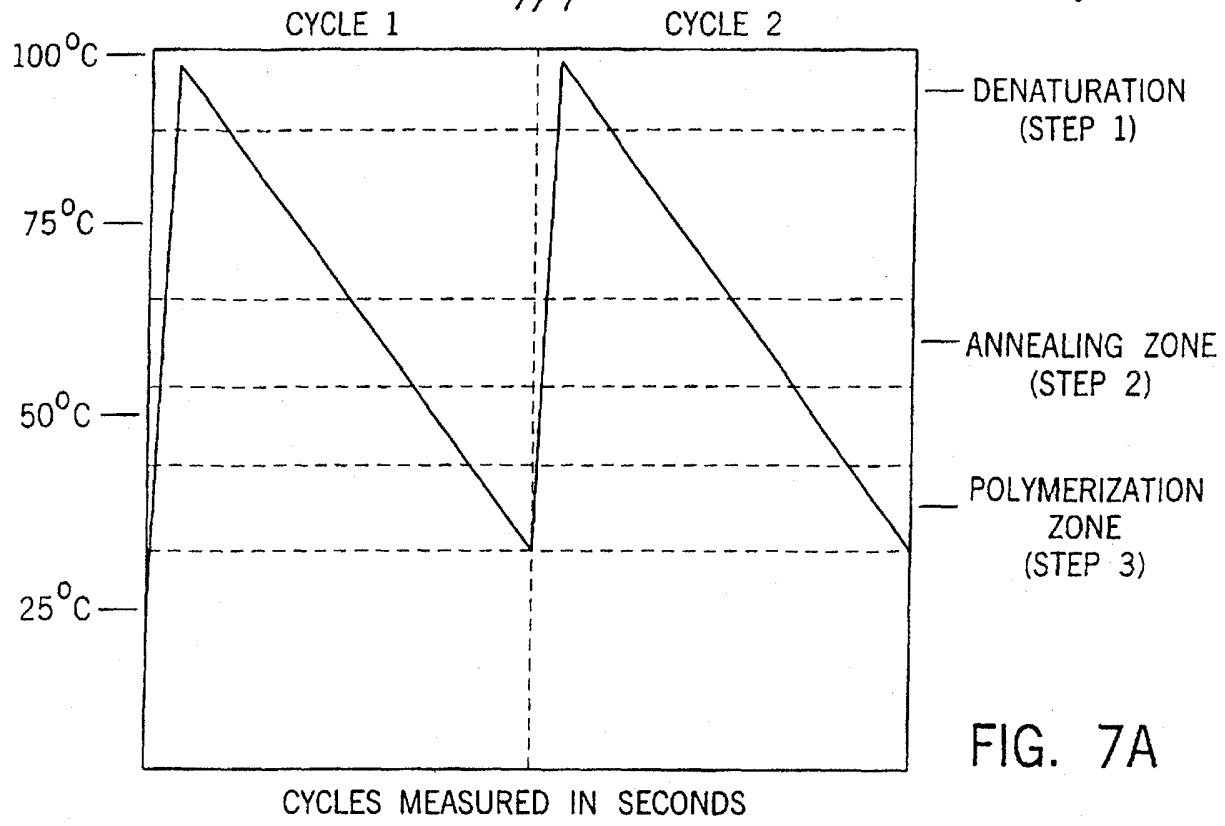
POLYMERIZATION ZONE (STEP 3)

FIG. 6B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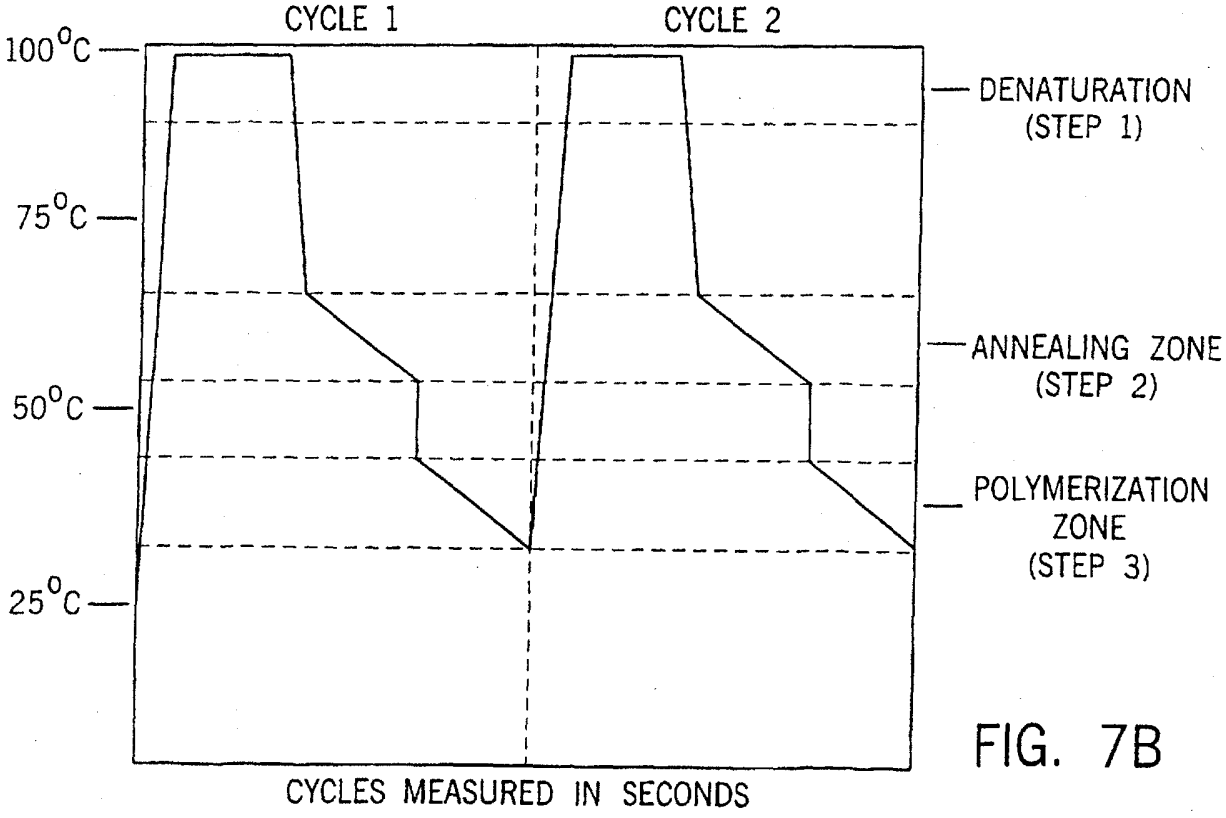
POLYMERIZATION ZONE (STEP 3)
CYCLES MEASURED IN SECONDS

FIG. 7A


CYCLE 1
CYCLE 2
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DENATURATION (STEP 1)
ANNEALING ZONE (STEP 2)
POLYMERIZATION ZONE (STEP 3)
CYCLES MEASURED IN SECONDS

FIG. 7B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/14307

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C12M 1/42; G01N 27/00; H01F 1/00

US CL :435/4, 6, 91.2; 285.2; 204/157.15, 403; 252/519

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 6, 91.2; 285.2; 204/157.15, 403; 252/519

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, 4,690,130 A (S.G. MIRELL) 01 September 1987, columns 3 and 8.	6 ----- 7-10
X --- Y	US 5,411,730 A (KIRPOTIN et al) 02 May 1995, columns 4, 15 and 16.	6 ------ 7-10
X	US 5,507,967 A (FUJITA et al) 16 April 1996, columns 4 and 9.	6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 SEPTEMBER 1997

Date of mailing of the international search report

22 OCT 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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INTERNATIONAL SEARCH REPORT

International application No. PCT/US97/14307

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS; DIALOG: BIOSIS, CA, DERWENT PATENTS, EMBASE, SCISEARCH, MEDLINE

search terms: electromagnetic or magnetic, radio frequency, RF responsive, solid, support, device, apparatus, temperature cycling or modulator, ferromagnetic, iron oxide, chromium dioxide, magnetite